

Substituted Guanidine Compounds as Inhibitors of Nonenzymatic Glycation *in vitro*

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Pathologic effects of the process of nonenzymatic glycation are reflected in degenerative changes during ageing, chronic complications of diabetes mellitus and renal failure, and have also been recognised in some neurologic diseases, such as Alzheimer's disease. Aminoguanidine has been extensively studied as an inhibitor of nonenzymatic glycation, both *in vitro* and *in vivo*. We investigated the inhibiting potency of substituted guanidines in the process of glycation. For this purpose, α -methylguanidine-acetic acid (creatine) and dimethylbiguanide (Metformin) were chosen. A common feature of these compounds is the presence of guanidine group in the molecule. Creatine is a specific muscle tissue metabolite, a non-toxic biogenic substance. Dimethylbiguanide is a substituted molecule of guanidine structure. In clinical practice, it is used in the treatment of non-insulin dependent type of diabetes mellitus. Both agents, α -methylguanidine-acetic acid and dimethylbiguanide, tested at concentrations of 2.5, 5, 10 and 20 mmol L⁻¹, showed a concentration dependent inhibition of the glucose induced albumin glycation *in vitro*. The inhibiting effect of substituted guanidines was somewhat inferior (17%) to the effect of aminoguanidine inhibition (52%); however, the former substances are valuable for being safe for human use.

Key words: nonenzymatic glycation, inhibition, creatine, dimethylbiguanide.

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INTRODUCTION

The ability of reducing sugars to react with amines as well as with basic amino groups of proteins and nucleic acids without enzyme mediation is a phenomenon known as nonenzymatic glycation. This is a classic covalent reaction whereby a sugar-protein complex is formed by *N*-glycoside linkage. The reactions of nonenzymatic glycation were described by Maillard, a French chemist, and have thus been named after him. Maillard's reactions have been extensively studied in food chemistry, however, it was only in the '70s that they were detected to occur also *in vivo*.¹ In physiologic conditions, nonenzymatic glycation can be detected in the process of ageing, however, the reactions are considerably faster and more intensive in the pathophysiologic conditions (*e.g.*, diabetes mellitus) associated with a persistently elevated concentration of glucose.² The pathologic effects of nonenzymatic glycation are reflected in chronic complications of diabetes mellitus, renal failure, degenerative changes occurring in the course of ageing, and have also been identified in some neurologic diseases.³

Maillard's reactions are very complex and multilevel, and can be analysed in three stages. In the first stage, glycosamine, *i.e.* unstable Schiff's base, is formed. This is followed by cyclisation and isomerisation, known as Amadori's rearrangement, whereby the compound 1-amino-deoxyketose, frequently called Amadori's compound, is formed. It is an early product of nonenzymatic glycation; an intermediary acting as a precursor of all compounds subsequently formed in the process of glycation.^{1,4} The second stage or intermediary stage reactions include formation of numerous intermediary products. Amadori's compound can be degraded by the reaction of autocatalysis, and by dehydrations, oxidation, cleavage or rearrangements, when 1,2 or 2,3 enols and a number of secondary products, such as aldehydes, dicarbonyls and reductones, are formed from the carbohydrate moiety. These diverse intermediaries repeatedly participate in further steps of the reaction by forming various compounds.⁴ The third, end stage includes complex reactions of polymerisation of the products formed in the second reaction stage, whereby heterogeneous structures commonly termed advanced glycation endproducts (AGE) are formed.² Some of the second-stage intermediary products enter the end stage of Maillard's reaction, when very complex polymerisations occur to form complex AGE structures. AGE formation can be very conveniently illustrated by means of 3-deoxyglucosone, which is formed by Amadori's compound deamination. Deoxyglucosone is just one of the intermediary products formed in the cascade of Maillard's reactions. However, it is an important intermediary because of its high reactivity,⁴ since it further reacts with terminal nucleophilic amino groups of lysine or arginine, thus forming complex polymers that contain pyrrolidine and imidazolone structures. Generally, AGEs are characterised by great structural and physicochemical diversity.

Thus, some AGEs are fluorescent polymers, *e.g.*, pentosidine, crosslines and imidazolones, whereas others are neither fluorescent nor have a reticulated structure, *e.g.*, pyrraline, *N*^ε-lactatolysine and *N*^ε-(carboxymethyl) lysine.⁴

Attempts have been made to pharmacologically influence the process of nonenzymatic glycation in order to prevent or slow down the formation of glycation products. There are two main pharmacological approaches. One includes inhibition of the rearrangement of early into endproducts of glycation, so as to prevent the rearrangement of early, still reversible compounds into the end, irreversible and crosslinked products.⁵ The other approach is based on the attempted cleavage of the already formed AGE products. The best-known AGE breaker is phenacyl thiazole bromide (PTB), which can break covalent bonds of crosslinked AGE products. AGE breakers have currently been intensively investigated.⁶

Aminoguanidine hydrochloride, a nucleophilic hydrazine compound, contains a terminal amino group, which is of higher chemical reactivity than terminal amino groups of proteins. For this feature, aminoguanidine has been chosen as an inhibitor of glucose binding to proteins. Aminoguanidine has been found to inhibit formation of AGE products, primarily by reacting with Amadori's products, *i.e.* by blocking the carbonyl groups on ketoamines and their derivative.⁷ Besides, aminoguanidine reacts specifically also with AGE peptides and other reactive intermediaries, thus preventing their binding to free amino groups. Numerous animal studies have shown that aminoguanidine might prevent or at least slow down the development of late complications in diabetes mellitus.^{8,9} It compromises collagen crosslinking; acts on vascular changes in the retina, peripheral nerves, aorta, kidneys and skin; decreases albuminuria; and reduces early structural changes in the vascular system and electrophysiologic changes in peripheral nerves. It has an inhibitory effect on precipitated development of diabetic retinopathy and formation of thrombi. Also, aminoguanidine decreases the content of AGE in the glomerular basal membrane and prevents its thickening. According to recent studies, aminoguanidine inhibits the activity of NO synthase. Although quite encouraging data on the effect of aminoguanidine have been reported by previous studies, clinical trials have not yet yielded as promising results as expected.

In the present study, we have tried to evaluate the potency of substituted guanidines, *i.e.* methylguanidines, as nonenzymatic glycation inhibitors. We used α -methylguanidine-acetic acid (creatine) and dimethylbiguanide (Metformin). A common feature of these compounds is the presence of guanidine group in the molecule (Figure 1). Creatine is a specific muscle tissue metabolite, a non-toxic biogenic substance. Dimethylbiguanide is a substituted molecule of guanidine structure, used in clinical practice for the treatment of non-insulin dependent diabetes mellitus.¹⁰

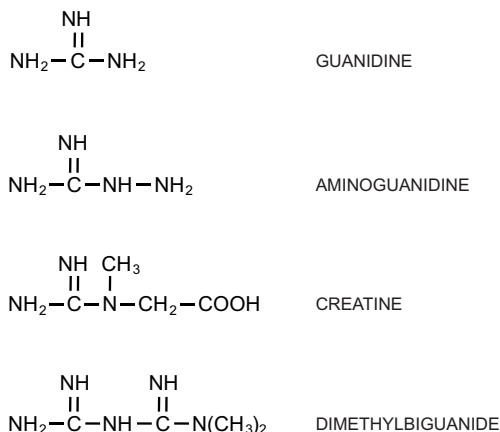


Figure 1. The structure of guanidine, aminoguanidine, creatine and dimethylbiguanide.

MATERIAL AND METHODS

Bovine serum albumin (Sigma: A-7906), creatine anhydrous, 1,1-dimethylbiguanide hydrochloride, aminoguanidine bicarbonate, D,L-arginine and L-lysine were purchased from Sigma, St Louis, USA. D-Glucose and all other chemicals were from Merck, Darmstadt, Germany.

Glycated protein was prepared *in vitro* by incubation of bovine serum albumin (BSA) and D-glucose. BSA was dissolved in 0.2 mol L⁻¹ phosphate buffer, pH 7.4, in a concentration of 10 mg mL⁻¹ containing 3 mmol L⁻¹ NaN₃, 5 mmol L⁻¹ EDTA-Na and penicillin-VK 100 U mL⁻¹. The reaction mixture was sterilised by filtration (0.45 µm pore filter) and allowed to incubate in dark at 37 °C for up to 21 days. Formation of glycated products was monitored by fluorescence spectrophotometry, on a daily basis continuously for three weeks. Fluorescence values of the samples were measured at a protein concentration of 5–10 mg mL⁻¹, and expressed in arbitrary units of fluorescence *per* mg protein. All readings were corrected for the blank value recorded in the preparation of albumin, incubated in the absence of D-glucose.

In the experiments on the potential inhibitory action, aminoguanidine, dimethylbiguanide, and creatine were incubated in a reaction mixture of albumin and glucose. The inhibitory effect of creatine was tested at concentrations of 2.5, 5, 10 and 20 mmol L⁻¹. Dimethylbiguanide was tested at concentrations of 5 and 10 mmol L⁻¹, and aminoguanidine at 2.5, 5 and 10 mmol L⁻¹. Reaction mixtures were sterilised by filtration (0.45 µm pore filter) and allowed to incubate in dark at 37 °C. Fluorescence intensity was measured in 1 mL of incubation mixture dissolved with 1 mL phosphate buffer, pH 7.4. Relative fluorescence was recorded on a Perkin-Elmer LS-5B luminescence spectrometer at a) 440 nm upon excitation of 370 nm for Maillard products related fluorescence, and b) 335 nm excitation and 385 nm emission for pentosidine-like products. A native BSA preparation (1 mg mL⁻¹ in phosphate buffer,

pH 7.4) was used as reference, and its fluorescent intensity was defined as one unit of fluorescence. The result was expressed in arbitrary units representing measured fluorescence *per* mg BSA (FU mg⁻¹). Measurements were made in comparison with appropriate blanks of 0.2 mol L⁻¹ phosphate buffer, pH 7.4, containing bovine serum albumin (BSA) alone, as well as a separate individual blank for each of the tested inhibitors. The fluorescence level of samples was corrected against the fluorescence of complete reagent blanks for BSA plus the tested inhibitors in increasing concentrations but without glucose.

Protein content was determined in each sample according to Bradford. Protein determination by this micromethod is based on protein reaction with Coomassie Brilliant Blue G-250. Upon stain binding to the protein, the colour changes from red to blue, *i.e.* maximal absorption changes from 465 to 595 nm.

RESULTS

Glycated albumin was prepared *in vitro* by incubation of BSA with glucose at concentrations of 20 and 100 mmol L⁻¹. During 21-day incubation, fluorescence of the reaction mixtures was measured at regular intervals on days 0, 7, 14 and 21, at excitation/emission of 335/385 and 370/440 nm. Formation of fluorophore was linearly dependent on the reaction time and sugar concentration.

The inhibitory effect of creatine on nonenzymatic glycation of albumin (with 20 mmol L⁻¹ glucose) was assessed upon addition of creatine to the reaction mixture, in concentrations of 5 and 10 mmol L⁻¹. At the beginning of the experiment and on day 7 of incubation, the level of fluorescence of incubation mixtures with creatine was 28 FU mg⁻¹, measured at ex/em 370/440 nm. On day 14, a slight fluorescence increase to 37.8 ± 0.7 FU mg⁻¹ was recorded, however, without any major difference between the samples with 5 and 10 mmol L⁻¹ creatine. On day 21, the fluorescence of the reaction mixtures containing 5 and 10 mmol L⁻¹ creatine was 72.2 ± 3.6 and 67.7 ± 3.1 FU mg⁻¹, respectively, the latter being significantly lower ($p < 0.001$) than the fluorescence of the control reaction mixture of albumin and glucose without addition of guanidine compound (81.6 ± 2.9 FU mg⁻¹). These data suggest that creatine could prevent formation of fluorescent compounds formed in the non-catalysed reaction of albumin and glucose. The inhibitory effect of 5 and 10 mmol L⁻¹ creatine was 11% and 17%, respectively. The inhibitory effect of creatine was analysed in a wider concentration range of 2.5, 5, 10 and 20 mmol L⁻¹ after 16 days of incubation. Fluorescence of the albumin and glucose reaction mixture without addition of creatine was taken as a measure of 100% glycated albumin formation. A 10% inhibitory effect was observed for creatine concentrations of 2.5 and 5 mmol L⁻¹, while its concentration of 10 mmol L⁻¹ showed a stronger, 14% inhibitory effect. Creatine

concentration of 20 mmol L⁻¹ produced the strongest inhibitory effect on fluorophore formation, reducing it by 35% (Figure 2). Accordingly, the inhibitory effect of creatine was found to depend on the concentration used. The effect of creatine (10 mmol L⁻¹) on nonenzymatic glycation of bovine albumin with a very high glucose concentration of 100 mmol L⁻¹ was tested as well. Fluorescence was measured at the beginning, and on day 14 and 21. The level of fluorescence of the reaction mixture of albumin and glucose showed a tendency to steady increase. At the same time, the fluorescence of the albumin and glucose mixture with addition of creatine was also observed to rise, however, at a slower rate. At the end of the experiment, fluorescence of 76.9 ± 0.5 FU mg⁻¹ was measured for the albumin and glucose mixture with addition of creatine, which was significantly lower ($p < 0.01$) than the fluorescence of the albumin and glucose reaction mixture without inhibitor addition. Comparison of the inhibitory effect of 10 mmol L⁻¹ creatine on albumin glycation in incubation with 20 and 100 mmol L⁻¹ glucose indicated that the increase in the concentration of glucose had no linear effect on the process of nonenzymatic glycation. Thus, the inhibitory effect of creatine, measured as fluorescence reduction, ranged between 15% and 20% in both cases of albumin glycation.

The effect of dimethylbiguanide on nonenzymatic albumin glycation is presented in Figure 3. Dimethylbiguanide in concentrations of 5 and 10 mmol L⁻¹ was incubated for three weeks in mixtures of albumin and 20 mmol L⁻¹

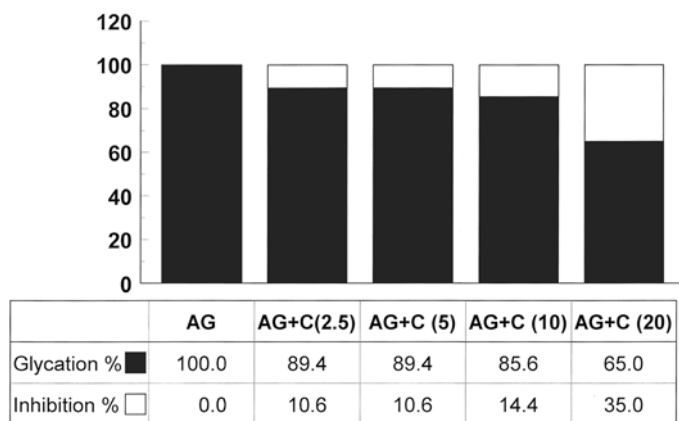


Figure 2. The inhibitory effect of creatine on the formation of fluorescent products (measured at 370/440 nm ex/em) of noncatalysed glycation after 16-day incubation of reaction mixtures containing albumin (A 10 mg mL⁻¹), glucose (G 20 mmol L⁻¹) and creatine (C) in concentrations of 0, 2.5, 5, 10 and 20 mmol L⁻¹.

AG = albumin + glucose; AG+C = albumin, glucose + creatine (conc.).

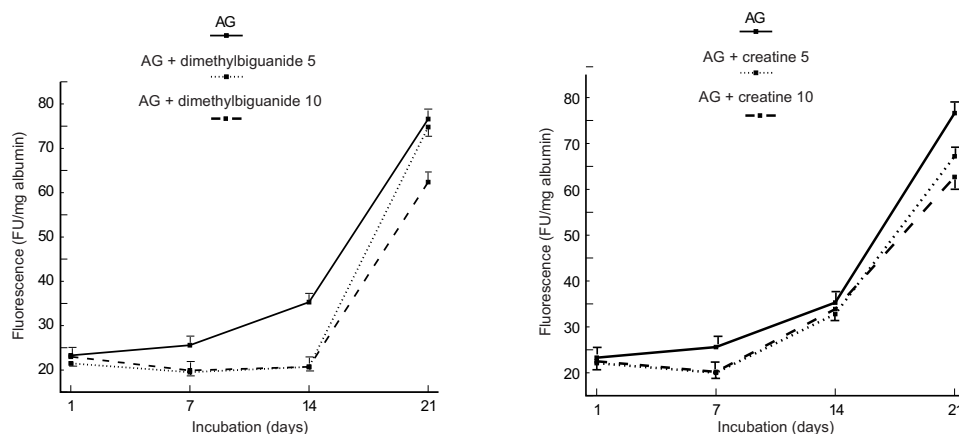


Figure 3. Nonenzymatic glycation derived fluorescence during 21-day incubation of reaction mixtures consisting of albumin (A 10 mg mL^{-1}), glucose (G 20 mmol L^{-1}) and dimethylbiguanide or creatine, both in concentrations of 5 and 10 mmol L^{-1} . Results are presented as the mean (\pm SD) of 10 measurements. Relative fluorescence was recorded at 440 nm upon excitation of 370 nm .

AG vs AG + dimethylbiguanide 5 mmol L^{-1} , NS.

AG vs AG + dimethylbiguanide 10 mmol L^{-1} , $p < 0.001$.

glucose. On day 7, the reaction mixtures with either dimethylbiguanide concentration showed no change from the initial fluorescence value. On day 14, the sample with 5 mmol L^{-1} dimethylbiguanide retained the same level of fluorescence, and on day 21 it increased to $79.6 \pm 8.0 \text{ FU mg}^{-1}$. With this increase, the measured fluorescence approached the value of $81.6 \pm 2.9 \text{ FU mg}^{-1}$, measured in the mixture of albumin and glucose without inhibitor addition. On day 14, parallel incubation of albumin and glucose with 10 mmol L^{-1} dimethylbiguanide showed a fluorescence intensity of 39.5 ± 6.5 ($p < 0.05$ vs AG), with a tendency to further increase. At the end of the experiment, on day 21, fluorescence of $67.4 \pm 6.7 \text{ FU mg}^{-1}$ was recorded, which was significantly lower than the fluorescence of the albumin and glucose mixture ($p < 0.001$) without addition of a potential inhibitor. These results indicated the inhibitory effect of dimethylbiguanide to depend on the concentration used. On day 21, a dimethylbiguanide concentration of 5 mmol L^{-1} showed no major inhibitory effect, however, a two-fold concentration of 10 mmol L^{-1} dimethylbiguanide was effective and lowered the level of fluorescence by 17%.

Our studies to date have shown that creatine and dimethylbiguanide have an inhibitory effect on nonenzymatic glycation of protein. Aminoguanidine has been demonstrated to inhibit rearrangement of early into endproducts

of glycation. Therefore, our experiment was designed so as to measure in parallel the inhibitory potential of creatine, dimethylbiguanide and aminoguanidine. Albumin was chosen as the target molecule for nonenzymatic glycation. Glucose concentration of 20 mmol L⁻¹ corresponded to physiologic hyperglycemia. The mentioned inhibitors were individually added to the reaction mixtures of protein and sugar, each in a concentration of 10 mmol L⁻¹. The values of fluorescence measured after three-week incubation are shown in Figure 4. Aminoguanidine was found to be the most potent inhibitor. After two-week incubation, the samples containing aminoguanidine showed no fluorescence increase, whereas the other two inhibitors failed to be as efficient. However, in three weeks of incubation, characterised by intensive formation of nonenzymatic glycation endproducts, the following results were recorded: aminoguanidine inhibited 52% of the fluorescent product formation, while creatine and Metformin had a comparable inhibitory effect, so that both of these guanidine compounds decreased the level of fluorescence by 17%.

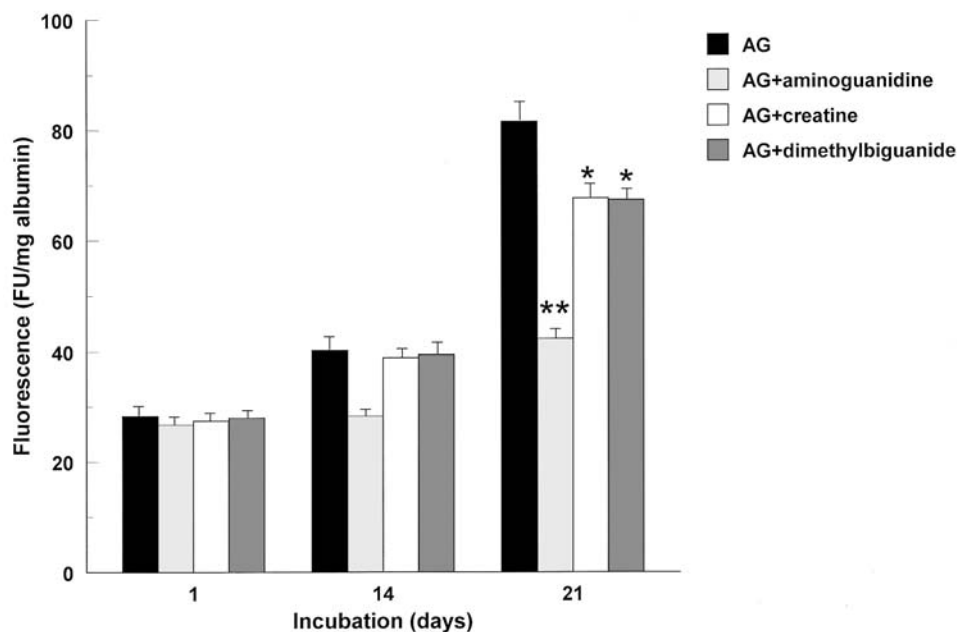


Figure 4. Comparison of the inhibitory effect of aminoguanidine, creatine and dimethylbiguanide (10 mmol L⁻¹ each) during nonenzymatic glycation of albumin (A 10 mg mL⁻¹) with glucose (G 20 mmol L⁻¹). Results are presented as the mean (\pm SD) of 10 measurements. Fluorescence was measured at a wavelength of 370/440 nm (excitation/emission). * $p < 0.001$; ** $p < 0.0001$.

DISCUSSION

Pharmacologic agents that specifically inhibit the process of nonenzymatic glycation have been mostly investigated in terms of delaying the development and progression of diabetes related complications. Aminoguanidine, a nucleophilic hydrazine of a guanidine structure, was the first and so far the best investigated agent. Aminoguanidine generally reacts with intermediary products of the second-stage Maillard's reaction that are not bound to protein structure, such as 3-deoxyglucosone.⁷ *In vitro* experiments have also demonstrated that aminoguanidine removes hydroxyl free radicals, and according to some authors, prevents lipid peroxidation *in vivo*. There is ample evidence confirming that the treatment with aminoguanidine in experimental diabetes reduces the micro- and macrovascular disease.⁸ However, due to its side effects, there seem to be some problems with the use in humans. Phase II/III clinical trials, conducted for some time now, have not yet produced any promising report.¹¹ This has stimulated us to embark upon this study, to see whether some other guanidine substances might have an inhibitory effect in the process of glycation. We chose α -methylguanidine-acetic acid (creatine) and dimethylbiguanide (Metformin) containing a guanidine structure in its molecule and with a substituted molecule moiety. These compounds were chosen primarily for their possible use in humans. Thus, α -methylguanidine-acetic acid or creatine is a biogenic guanidine compound found in muscular tissue, where it is involved in the formation of high-energy ATP.¹² There are also reports on its hypoglycemic action.¹³ Like aminoguanidine, creatine has a terminal nucleophilic amino group. Dimethylbiguanide is a substituted molecule of a guanidine structure, which was years ago demonstrated to have a hypoglycemic effect. Dimethylbiguanide or Metformin has been widely used in clinical practice for the treatment of non-insulin dependent diabetes mellitus. Results of a recent study suggest that Metformin is efficient in scavenging reactive carbonyls, the intermediaries produced during Maillard's reactions.^{14,15}

During *in vitro* and *in vivo* nonenzymatic glycation of proteins, numerous products are formed, which are characterised by great structural and physicochemical diversity. The great heterogeneity of glycated products poses considerable difficulties in the attempts at their qualitative and quantitative determination. In the present study, we measured the glycation product-derived fluorescence as a palliative parameter, yielding a relative difference in the intensity of nonenzymatic glycation. In all our experiments, we used bovine serum albumin as a target molecule of nonenzymatic glycation at high glucose concentrations in semiphysiologic conditions and identical *in vitro* glycation conditions. The results indicated both creatine and Metformin to be potential inhibitors in the process of nonenzymatic glycation. The effi-

ciency of inhibition depended on the guanidine substance concentration, so a more potent inhibitory effect was obtained by increasing the concentration of creatine (2.5, 5, 10 and 20 mmol L⁻¹). The most potent inhibition of 35% was measured with a creatine concentration of 20 mmol L⁻¹. Dimethylbiguanide showed a similar pattern. A concentration of 10 mmol L⁻¹ lowered the level of fluorescence in the incubation mixture by 17%. It should be noted that similar rates of inhibition were invariably recorded at various glucose concentrations. The increase in the glucose level from 20 to 100 mmol L⁻¹ did not reduce the inhibitory action of creatine. Even at an artificially high glucose concentration of 100 mmol L⁻¹, the inhibitory effect of 5 and 10 mmol L⁻¹ creatine remained the same, *i.e.* between 15% and 20%, thus definitely confirming our preliminary results.¹⁶ However, the effect of inhibition with substituted guanidines, creatine and Metformin, was considerably lower than that recorded with aminoguanidine. This comes as no surprise, because the structure of substituted guanidines reveals that they have fewer nucleophilic terminal amino groups. In spite of this, the results presented should not be disregarded, primarily because the substances tested in this study are safe for human use, and it may prove plausible to evaluate them in a clinical trial. It has been long known that muscular activity improves the tolerance of glucose load, and that the level of blood glucose decreases with exercise. As creatine is a specific muscle tissue metabolite, its synthesis being intensified with enhanced muscular activity, we could speculate on the possible involvement of the inhibitory mechanisms of nonenzymatic glycation in this phenomenon.

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SAŽETAK

Supstituirani guanidini kao inhibitori u neenzimskom glikoziliranju *in vitro*

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Patološki učinci što ih proces neenzimskog glikoziliranja potiče *in vivo* ogledaju se tijekom starenja u komplikacijama šećerne bolesti, bubrežnoj insuficijenciji, a zabilježeni su i u nekim neurološkim bolestima. Aminoguanidin je bio prvi i do sada najbolje proučavan inhibitor Maillardovih reakcija. No *in vivo* su zapažena i njegova štetna djelovanja. Stoga se u ovom radu željelo utvrditi mogu li supstituirani guanidini, koji nisu štetni za humanu primjenu, inhibirati reakcije neenzimskog glikoziliranja. U tu su svrhu izabrani α -metilguanidino-oktenu kiselina (kreatin) i dimetilbiguanid (Metformin), jer oba spoja uključuju guanidinsku strukturu. Kreatin je specifičan mišićni metabolit, dakle netoksična biogena supstancija. Dimetilbiguanid je supstituirana molekula guanidinske strukture, a u kliničkoj se praksi koristi kao oralni hipoglikemik. Oba su spoja primijenjena u koncentracijama 2,5; 5; 10 i 20 mmol L⁻¹, pri semifiziološkom inkubiranju ljudskog albumina s glukozom i pokazali su inhibicijski učinak. Zabilježena relativna inhibicija (17%) bila je niža od učinka aminoguanidina (52%).